EVALUATION OF CYTOCOMPATIBILITY OF PLGA AND PLGA-BASED NANOCOMPOSITE BIOMATERIALS IN OSTEOBLAST CULTURES

A. Ścisłowska-Czarnecka[1], E. Stodolak-Zych[2], E. Prawdzik[3], M. Boguń[4], E. Menaszek[2,3]

[1] University School of Physical Education, Faculty of Rehabilitation, Physiotherapy Department, al. Jana Pawła II 78, 31-571 Krakow, Poland
[2] AGH University of Science and Technology, Faculty of Materials Science and Ceramics, Department of Biomaterials, al. A. Mickiewicza 30, 30-059 Krakow, Poland
[3] Jagiellonian University, Collegium Medicum, Department of Cytobiology, ul. Medyczna 9, 30-688 Krakow, Poland
[4] Technical University of Lodz, Faculty of Material Technologies and Textile Design, Department of Man-Made Fibres, ul. Żeromskiego 116, 50-952 Lodz , Poland

Abstract

The aim of this study was to evaluate biocompatibility of multilevel composites based on bioresorbable poly(lactide-co-glycolide) (PGLA). Polymer matrix was modified with multidimensional (MD) short biopolymer fibers of calcium alginate (CA) containing bioactive ceramic nanoparticles (nanohydroxyapatite - HA or nanosilica - SiO_2). The nanocomposite fibres present in the polymer samples influenced cells morphology, viability and secretory activity which was estimated using human osteoblasts cells (NHOst). The results indicate that biodegradable nanocomposite CA-HA/PGLA improves biological properties of the basic biomaterial (PGLA) suggesting its potential application for bone tissue engineering.

Keywords: *cells materials interaction, cells factors, composite, nanocomposite materials*

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Introduction

Polymer-fiber composites are one of the main groups of materials used in the bone tissue regenerative medicine, which have better utilitarian properties than currently used implants [1-5]. One of the most promising approaches in modern medicine is make the most of regenerative potential of living organism. As a support for defected tissue regeneration nanocomposite materials were applied. In comparison to classic (bio)materials nanocomposites show better mechanical and biological properties [6]. Interestingly, combination of two biocompatible resorbable polymer together with other bioactive materials e.g. fibers or powder [7] gives the possibility to obtain biocomposite materials with properties which mimic natural extracellular substance of the bone (structurally biomimetic). Moreover, this kind of multilevel composite materials (in macro level – fibres, in nano level - nanoparticles) can be formed as two- or three-dimensional materials (films, membranes or scaffolds) to create optimal conditions for multicellular culture for the bone regeneration.

In the present work hybrid fibrous supports based on biodegradable co-polymer of glycolide and L-lactide (PGLA) containing nanocomposite calcium alginate fibres (CA) enriched with either nanometric hydroxyapatite (CAH) or nanometric silica (CAS) were prepared. The aim of application of biopolymer fibres made of calcium alginate was the fabrication of biomimetic polymer-fibrous structure which simultaneously was a reservoir of bioactive particles. Nanocomposite fibres were comminuted to submicron and micron sizes and introduced into the PGLA polymer matrix. Preliminary studies showed that CAS/PGLA and CAH/PGLA hybrid materials were biocompatible and their surface properties positively influenced adhesion of bone cells. Potential site of implantation of the studied materials is bone tissue and thus their effect on morphology and secretory activity of osteoblasts, which are crucial cells for its development and rebuilding, was tested.

Materials and Methods

Materials in the form of 2D supports (thin foils) were produced using the casting method. As matrix was used poly(lactide-co-glycolide) (PGLA, Sigma-Aldrich) which was dissolved in dichloromethane (CH_2Cl_2, POCh) in 1:10 wt. ratio. As nanocomposites fibrous phases were used short biopolymer fibres (calcium alginate, FGM BioPolymers) modified by nanometric silica (5-10 nm, Sigma Aldrich) and nanometric hydroxyapatite (30-50 nm, Sigma-Aldrich). Nanocomposite fibres (calcium alginate with silica – named CAS and calcium alginate with hydroxyapatite – named CAH) were produce by wet method in Technical University of Lodz. Before the introduction into the matrix the nanocomposite fibres (CAS, CAH) were mechanically ground in a vibration ball mill. The final form of the modifier was a mixture of two fractions; short fibres, particles of the fibres and the nano-additive, which were products of the nanocomposite fibres fragmentation. The mean size of the fibres fragmentation products (biopolymer particles, nanoadditives) was between 20 and 30 μm (optical microscope). The polymer – fibres nanocomposites (2 wt% of nanocomposite fibres) were prepared using a casting method. Characteristic of thin foils PGLA/CAS and PGLA/CAH were presented in previous study [8].

Cell cultures

NHOst osteoblast cells (Clonetic Normal Human Osteoblast Cell System, Lonza, USA) were cultured in 75-ml plastic bottles (Nunc, Denmark) in OGM culture medium (Lonza, USA) enriched with ascorbic acid (Lonza, USA), 10% fetal bovine serum (Lonza, USA) and 100 U/ml penicillin 10 UI/mL and 100 μg/mL streptomycin (Lonza, USA). Cells were cultured in the incubator HeraCell (ThermoScientific, Germany) at 37°C and 5% of CO_2. Every 3 days the cell cultures were passaged by trypsinization (0.25% solution of trypsin, Sigma-Aldrich, Germany). Before cell culture the biomaterial foils were washed in 70% ethanol and sterilized under UV lamp for 30 min from both sides and placed at the 24-well culture dishes (Nunc, Denmark). Cells (after 3 passages) were counted in Bürker's hemocytometer, and seeded ($1.5x10^4$ cell/ml) in the 24-well culture plates (Nunc, Denmark) containing foils of the tested biomaterials. Pure PGLA foil was used as a control (CTR) material. Cells were cultured for 3 or 7 days. Subsequently, morphology of cells adhering to the biomaterials was observed using the inverted microscopy. Supernatants from cell cultures were collected and frozen at -20°C prior to cytotoxicity, protein and nitric oxide evaluation.

Cell morphology and viability

Cells were stained for 2 min with 0.01% acridine orange (Sigma-Aldrich, Germany) in PBS, washed with PBS, and observed using the fluorescent microscope (Olympus CX41, Japan).

Cytotoxicity assay

The loss of cell membrane integrity is reflected in the release of adenylate kinase AK, which can be detected using the firefly luciferase system (ToxiLight® BioAssay Kit, Lonza, Basel, Switzerland), a highly sensitive assay designed to measure cell membrane damage [9]. After 3 and 7 days of osteoblast incubation on the tested biomaterials, 5 µl of culture media was transferred to wells of a 384-well plate (Nunc, Denmark) and 25 µl of the Adenylate Kinase Detection Reagent (AKDR) were added per well. The plates were incubated for 5 min in the dark, and then luminescence was measured using the luminometer (PolarStar Omega) at 565 nm. Culture medium served as negative control, while cells treated with active AKDR reagent were used as positive control.

Nitrite/nitrate (NO) production

The total amount of NO (NO_2^- and NO_3^-) was measured as described previously [10]. Briefly, nitrate was reduced to nitrite by addition of nitrate reductase, FAD and NADPH (all from Sigma-Aldrich, Germany) and then NADPH was oxidized by lactate dehydrogenase in the presence of sodium pyruvate (both from Sigma-Aldrich, Germany). Finally, nitrite concentration in the samples was measured by the Griess reaction, by adding Griess reagents (0.1% naphthalethylenediamine dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_3PO_4; 1:1 vol/vol) in a ratio 1:1 to samples and standards. Sodium nitrite solution was used as an internal control for the Griess assay and $NaNO_3$ (both from Sigma-Aldrich, Germany) as controls for reduction step. The NO levels were measured at 540 nm with Expert Plus spectrophotometer (Asys Hitech, Eugendorf, Austria).

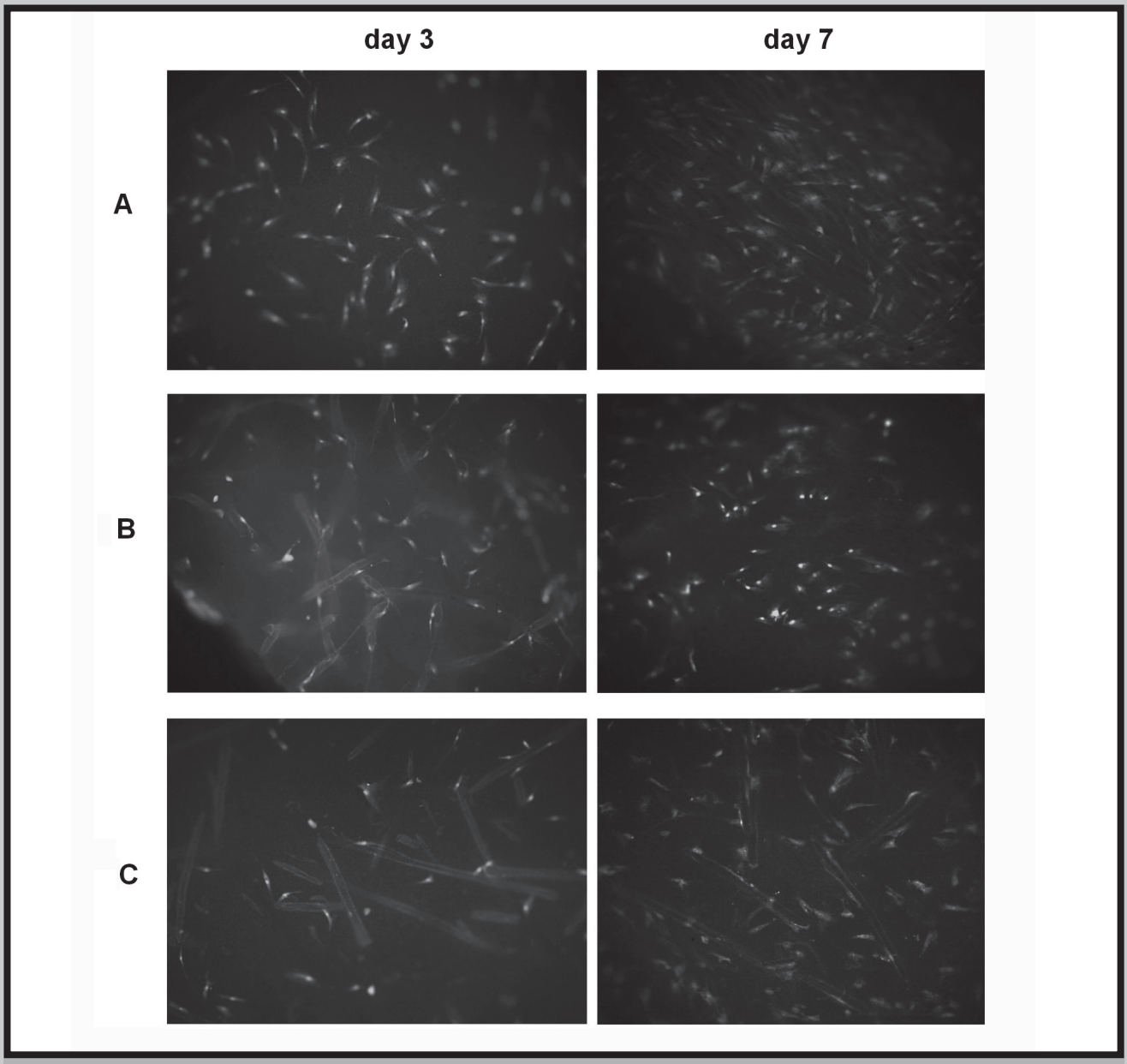

FIG. 1. Morphology of NHOst osteoblasts cultured on a control material PGLA and on PGLA including nanoadditives [CAS/PGLA or CAH/PGLA]. Morphology of cells was analyzed in fluorescence microscopy after 3 (left panel) and 7 days (right panel) of incubation. Analized materials: PGLA (A) CAS/PGLA (B), CAH/PGLA (C).

Protein production

Protein concentration in the supernatants collected from cell cultures was measured by the colorimetric BCA method as described previously [11]. Briefly, a mixture of copper (II) sulfate solution (CS, Sigma-Aldrich, Germany) and bicinchoninic acid solution (BCA; Sigma-Aldrich, Germany) in a ratio of 1:50 was prepared. Subsequently 10 µl of each tested sample was transferred to wells of a 96-well plate and then 200 µl of the CS/BCA mixture was added. The plates were incubated for 30 minutes in the dark. The optical density was measured at 570 nm with Expert Plus spectrophotometer (Asys Hitach, Austria).

Statistics

The results were presented as mean values +/- the standard error (SEM). Statistical analyses were performed using the T-Tukey's test. The statistical significance of differences was set at $p < 0.05$.

Results and discussion

Disruptions of the cell functions, their adherence, proliferation or secretory activity, may lead to delayed and/or incorrect integration of implanted biomaterial with recipient tissue. This can for example affect balance between bone formation and resorption reactions, which may then cause decrease of bone mass at the implantation site [12]. Although suitable for use in bone tissue regeneration physico-chemical properties of synthetic biodegradable polymers have been developed so far none of bioactive polymer culture medium does not satisfy the assumptions of the bone tissue engineering [13]. Therefore, in the present work we focused on examining the functioning of NHOst osteoblasts in vitro on polymer-fibre composites containing silica and hydroxiapatite nanoparticles. These nanoparticles gradually released from matrix supposed to improve osteoinducive or osteoconductive properties of biomaterials. We studied cell morphology, viability, and their secretory activity. On day 3 of culture the highest concentration of cells was observed on the surface of PGLA control material while fewer cells adhered to CAS/PGLA and CAHA/PGLA materials. Morphology of cells adherent to all materials was similar, osteoblasts adopted spindle shape (FIG. 1A). On day 7 number of the cultured cells increased on all materials in comparison to day 3, though the highest number of adherent osteoblasts was still observed on the control PGLA (FIG. 1B). Reduced osteoblast adherence/proliferation on materials with nanoparticles may be associated with their higher roughness. It was observed previously that smooth PGLA surface facilitates cell adherence and proliferation, whereas a rougher surface e.g. of PGLA modified by fibres hampers both processes [14-15]. However, same authors [9] showed that in vivo nanoparticles can form an anchor site for bone cells and therefore facilitate integration of implant. Both on day 3 and day 7 of culture a level of adenylate kinase (AK) in supernatant from osteoblasts cultured on CAS/PGLA was comparable to AK level measured in supernatants from cells cultured on control PGLA material (FIG. 2A and 2B), while it was lower in supernatants collected from cell cultures containing CAH/PGLA (FIG. 2A and 2B). These results are consistent with results of experiments obtained by Nie and co-workers [16], which showed that PGLA encapsulated with hydroxyapatite reduced the cytotoxicity of this material. Probably higher cytotoxicity of PGLA in vitro is the result of acidic pH of its degradation products. PGLA is yet successfully used as a material for implantation thanks to different regulation of system acidity changes in vivo [14]. Present results suggest that addition of CAH reduced cytotoxicity of the initial PGLA material.

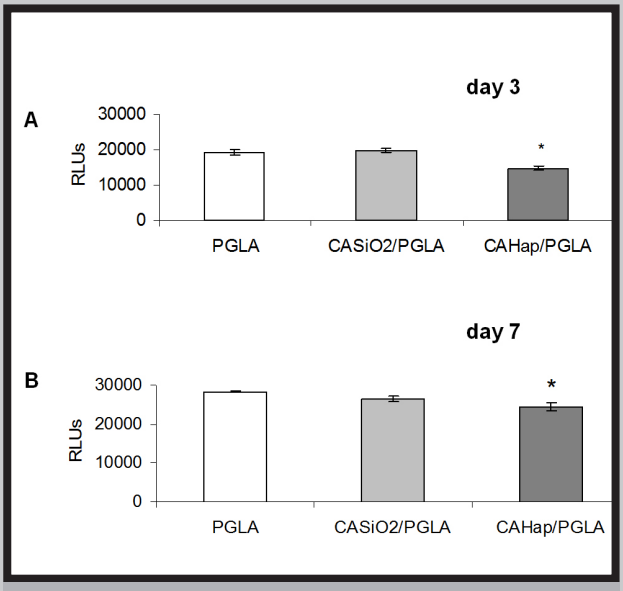

FIG. 2. Cytotoxic effect of control material PGLA and nanocomposites materials basing on PGLA on NHOst osteoblast. Cells were cultured on biomaterials either 3 (A) or 7 days (B).

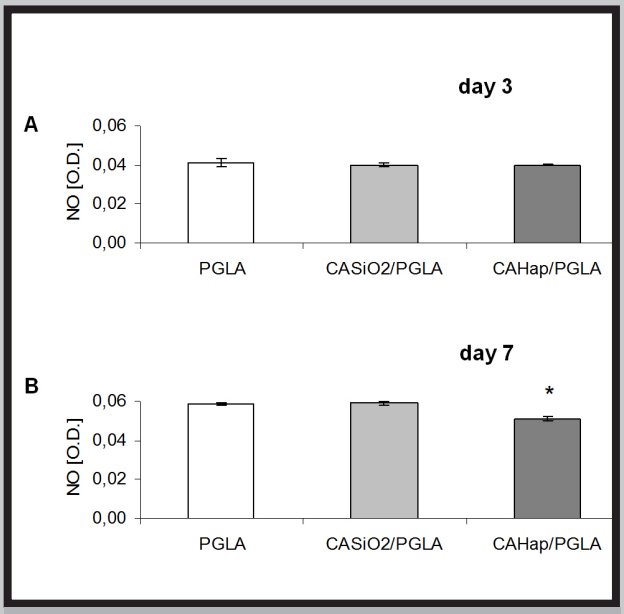

FIG. 3. Production/release of nitric oxide (NO) by NHOst osteoblasts cultured on PGLA and nanocomposites materials basing on PGLA. Cells were cultured on biomaterials either 3 (A) or 7 days (B). All results are shown as means±SE. * indicates values significantly different compared to the control by ($p<0.05$).

Interestingly, osteoblasts cultured on this material released to supernatant lower amounts of proteins (on day 3 and 7, FIG. 4A, FIG. 4B) and nitric oxide (day 7, FIG. 3B) compared to cells cultured on PGLA only. Nitric oxide (NO) is an important immune mediator with profound cytotoxic effects towards surrounding cells and tissues [17]. Consequently, NO serves as a good indicator of inflammation and should be monitored e.g. during the wound healing process [18]. Reduced secretion of proteins and NO by osteoblasts cultured on CAH/PGLA suggests that hydroxyapatite nanoparticles did not stimulate inflammatory reaction which can be a good predictor for the future in vivo studies.

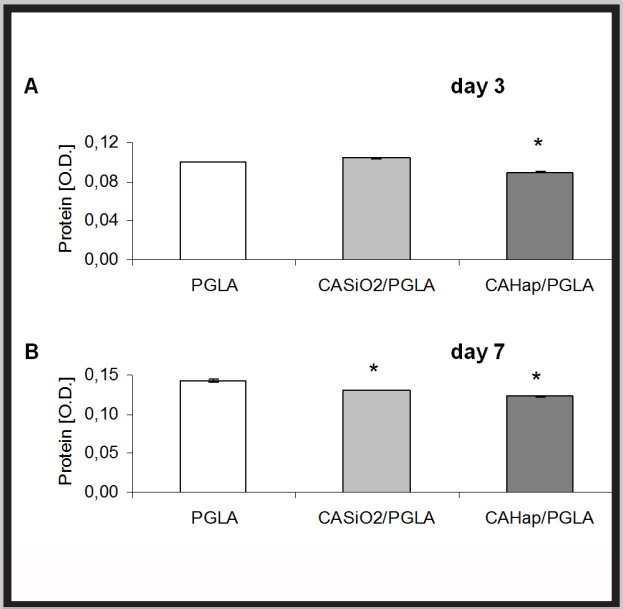

FIG. 4. Production/release of proteins by NHOst osteoblasts cultured on PGLA and nanocomposites materials basing on PGLA. Cells were cultured on biomaterials either 3 (A) or 7 days (B). All results are shown as means±SE. *indicates values significantly different compared to the control by ($p<0.05$).

Conclusions

The investigations demonstrated that the produced composite hybrid materials which were a multilevel composite consisting biopolymer fibers (CA) with nanoparticles (SiO_2, HA) can be applied in regenerative medicine. We can conclude that the addition of nanohydroxyapatite in biopolymer fibres to PGLA had a positive effect on osteoblast viability and activity, but more detailed studies are necessary to clarify its influence on bone formation.

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